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Antibacterial activity of selected essential oils against pathogenic bacteria causing human pyospermia

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ABSTRACT

Urogenital infections can impact the male reproductive system in various ways. The increasing resistance of microorganisms to conventional chemicals and drugs has prompted scientists to search for novel sources. In this study, among 186 human semen samples, 65 (34.94%) cultures were found to be positive for bacterial growth. All bacterial isolates were screened for antibiotic susceptibility tests. Regarding the antibiogram, all bacterial isolates showed high resistance to widely used antibiotics. In addition, the antimicrobial activity of three essential oils (cinnamon, lavender, and rosemary) showed strong inhibitory activity against *Enterococcus faecalis* (10.0 to 21.3 mm), *Staphylococcus haemolyticus* (9.7 to 34.3 mm), *Escherichia coli* (9.0 to 22.7 mm), *Micrococcus lylae*(17.0 to 39.7 mm), and *Serratia marcescens* (9.3 to 17.3 mm).As a good result, the MIC values of essential oils ranged from15.6 to 125µL/mL. Moreover, GC-MS analysis of the three essential oils revealed the presence of Linalool (49.45%), cinnamaldehyde, (E) (43.55%) and limonene **(**34.99%) as the predominant bioactive compounds in lavender, cinnamon and rosemary essential oils respectively. Most detected bioactive compounds demonstrate antimicrobial action by increasing cell permeability, causing cell leakage and reduced nucleoplasm. These findings confirm the antibacterial potential of selected three essential oils present in the current work are a good source of antimicrobial candidates that are effective against urogenital pathogenic bacterial infection.

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Introduction

Urogenital inflammation can impact the male reproductive system in several ways, inflammation that either directly or indirectly impacts sperm function and spermatogenesis. Urogenital tract infections can be caused by types of factors, including exposure to environmental contaminants, alcohol and tobacco use, specific drugs, and surgical procedures (Eini et al. 2021). Studies have shown that urogenital tract infections change the composition of seminal plasma, which obstructs the reproductive tract. Furthermore, an anti-sperm antibody is created when an infection or inflammatory process breaches the blood testis barrier, thereby impairing sperm's capacity to fertilize (**Babandi** *et al.* 2022). According to previous studies, aerobic cocci represented about fifty percent of semen samples of infertile patients. *Enterococcus faecails* isolates have been found in 53% of infected samples, while 20% were infected with *Micrococci*, and 16% were infected with alpha-haemolytic *Streptococci*. A higher frequency of *E. faecails*-caused reproductive tract infections is linked to lower-quality semen by alteration in shape and sperm concentration. Due to bacteria' growing resistance to traditional drugs, researchers are looking for new sources of broad-spectrum antimicrobial agents (Laws *et al.* **2019)**. Essential oils are used as flavoring agents and in aromatherapy in a wide range of items, including foods, drinks, cosmetics, and medications. They might be well-known for their therapeutic, antibacterial, aromatic, and antioxidant properties (Shaaban 2020). Previous research indicates that the antibacterial activity of EOs on human isolates can be used to prevent infections that are contracted in medical facilities or the community (Bolouri et al. 2022). Different component concentrations can affect the antibacterial potential of essential oils. For example, EOs possess antibacterial properties due to large concentrations of citral, eugenol, or cinnamic aldehyde (Angane et al. 2022). An example of the EOs' mechanism is a breakdown of the cell wall (Andrade-Ochoa et al. 2021), cytoplasmic coagulation and damage to the cytoplasmic membrane (Abdelhamed et al. 2022), damage to the membrane proteins, increasing permeability and allowing cell contents to escape (Yap et al. 2021). Strong antibacterial, antifungal, and antiviral qualities can be found in the phenols and monoterpenes found in essential oils of rosemary, lavender, and cinnamon (Elshafie et al. 2023; Nair et al. 2022). This may prove to be a successful strategy for slowing the development of antibiotic resistance and increasing the efficacy of conservative treatment choices (El-Tarabily et al. 2022; Kaōániová et al. 2020). This study aims to determine the antimicrobial potency of three essential oils against bacteria causing pyospermia.

Materials and Methods

Ethical approval

The study was approved by Al-Azhar University, Egypt (REC number: 00000400). This was done following the ethical standards of the 1964 Helsinki Declaration and its later comparable ethical standards or amendments, as well as the ethical standards of the national and/or the institutional research committee. The maneuver was explained, and written consent was taken from all couples before starting the study. This work included 180 human semen samples from unselected couples consulting an infertility clinic center. All subjects were attendants of the International Islamic Center for Population Studies and Research (IICPSR). ART unit, AL-Azhar University, Cairo, Egypt.

Semen specimen collection and Microorganisms

After two to seven days of abstinence, the semen specimens were obtained via masturbation while the environment was sterile. Before the collection of semen specimens, patients refrain from taking any antibiotics for a week. After the semen was liquefied within 60 minutes of collection, analysis was done. After the collection of samples, the experiments were completed in an hour. The inoculation media for semen samples were Blood agar, MacConky (Oxoid Ltd. Co., UK), and particularly chromogenic agar medium (Himedia, India). Following incubation, bacterial colonies were observed. After the recovered bacteria were purified and identified phenotypically with Gram staining and cell morphology. Standard microbiological techniques were followed in the biochemical identification of the isolates (Forbes et al. 2016).

Molecular identification by 16 d RNA PCR
DNA extraction

A technique based on sodium dodecyl sulfate (SDS) was used to extract DNA from bacterial colonies (Natarajan et al. 2016). A lysis buffer containing SDS, tris aminomethane hydrochloric acid (Tris HCl), and ethylene-diamine tetra-acetic acid (EDTA) was used to treat the bacterial cell suspension. Centrifugation was used in conjunction with a series of consecutive processes to remove cell debris and other contaminants. Ethyl alcohol that had been refrigerated was used to precipitate the genomic DNA. Using centrifugation, the precipitated DNA was collected as a pellet. After dissolving the pellet in TE buffer, it was kept at 40°C

until needed again. The universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′ TACGGTTACCTTGTTACGACTT-3′) were used according to Gumaa et al. (2021).

Amplification

PCR-based 16S rRNA gene amplification 50 µl was the entire reaction volume for the PCR experiment, which included 10 pmol of each primer (forward and reverse, bought from Sigma), 5 µl of DNA template, and 1U of Ampli Taq DNA polymerase. Each liter contained 200 µmol of deoxyribonucleoside triphosphate, 1.5 mmol of MgCl2, 10 mmol of Tris-HCl (pH 8.8), 50 mmol of KCl and 0.1% Triton X100. The PCR was conducted under the following conditions: a five-minute initial denaturation at 95°C; thirty-four cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min; and a five-minute final extension at 72°C. An agarose gel was used to run an aliquot (2 µl) of the PCR results to verify. An aliquot (2 µl) of PCR products was run in an agarose gel to check for amplified fragments.

Sequencing analysis

A QIAquick gel extraction kit (Sigma) was used to purify the PCR product. Using the ABI Prism 310 genetic analyzer (Applied Biosystems, USA), Big Dye Terminator Cycle Sequencing (version 3.0), and 15µl of the purified product, sequencing was done. Using an identification algorithm based on the selection of the longest recursive matches for the best alignment of the compared sequences, the sequence was compared to those in a reference database. Sequences from the NCBI GenBank reference database were obtained and merged. Manual comparisons were made between the final sequence and the best matches as described by Alsamman et al. (2023).

Antibiotics susceptibility test for isolated bacteria

The antibiotic susceptibility of each bacterial isolate was examined on Mueller-Hinton Agar (MHA) medium using the standard Kirby-Bauer disk diffusion technique, as defined by the CLSI (Clinical and Laboratory Standards Institute 2016).The antibiotics used in disc diffusion tests were, Chloramphenicol (30µg), Nalidixic acid (30µg), Nitrofurantoin (300µg), Rifampin (5µg), Amikacin (30µg), Cefuroxime (30µg), Clindamycin (2µg), Vancomycin (30µg), penicillin G (10µg), Tetracycline, (30µg), Levofloxacin (5µg), Gentamicin (10µg), Trimethoprim/suphamethoxazole (25µg), Imipenem (10µg), Erthromycin (15µg), Meropenem (10µg),Ciprofloxacin (5µg), and Piperacillin/Tazobactam (36µg). (Oxoid, UK).

Essential oils

Rosemary, lavender, and cinnamon oils, from the National Research Center (Egypt). Samples were obtained as pure essential oils using steam distillation. Glass vials with Teflon-sealed covers were used to keep the essential oil samples, which were kept in darkness and at room temperature.

GC–MS investigation

The volatile constituents were investigated via GC-MS (Agilent, USA; 7890A-5975C) using a capillary HP-5 column (the column was 0.25 µm thick, 0.25 mm inner diameter, and 30 meters in length) using a stationary phase consisting of a 95% methyl and 5% solution of dimethyl polysiloxane solution. A carrier gas of helium was applied at a 1.0 mL/min flow rate. 0.2 µL of injection was used, and the split ratio was 30:1. The oven was kept at 40 °C for five minutes, and then it was raised to 260 °C at a rate of 3°C/min and held for ten minutes. Finally, it was raised to 280°C at a rate of 10 °C/min and held for two minutes. The electron multiplier voltage was set to 1823.5 V, and the mass spectrometer was run in the electron ionization mode at 70 eV. The temperature of the quadrupole rod was used at 150 °C with a maximum temperature of 200 °C, and the ion source was set up at 230 °C with a maximum temperature of 250 °C. 50.0-550.0 amu was the mass range covered by this scan. The acquired mass spectra were compared to the reference mass spectra from the NIST11.L database to identify all volatile components (Youssef et al. 2021).

Screening for the antibacterial effect of essential oils

The antibacterial properties of three Eos, lavender, rosemary, and cinnamon were tested against bacterial isolates that cause pyospermia. The EOs were filtered through a 0.45 µm membrane filter after being diluted in 10% aqueous dimethylsulfoxide (DMSO) with Tween 80 (0.5% v/v for easy diffusion). Whatman discs measuring 6 mm were aseptically impregnated with 25 µL of varying concentrations (1:1, 1:2, and 1:4) of the corresponding essential oils. The discs were then placed on Mueller-Hinton sterile agar plates and inoculated with 10^8 CFU of bacterial strains (National Committee for Clinical Laboratory Standards 2002).Levofloxacin disc served as the negative control and amikacin disc as a positive control. To prevent inevitable evaporation of the contents, sterile laboratory parafilm was used to seal each Petri dish. After allowing the oil to diffuse for 30 minutes at room temperature, the plates were incubated for 18 hours at 37°C. The zone of inhibition was assessed following the incubation period. Three separate studies were conducted, and the mean value (± standard deviation) of the findings was reported.

Minimum inhibitory concentrations (MIC)

Each pure essential oil was added to 5 % dimethyl sulfoxide (DMSO). Serial dilutions of EO ranged from 500 to 3.9 µL/mL in test tubes containing Mueller–Hinton broth, each tube was inoculated with 100µL bacterial suspension adjusted to 10^8 CFU/mL. Tubes containing medium or essential oil alone were the negative control, and broth medium inoculated with bacteria was the positive control (Mahboubi & Kazempour 2009). The tubes were then incubated for 24 hours at 37°C. MIC values are defined as the lowest essential oil concentration that prevents observable bacterial growth.

Transmission electron microscopy

According to Tintino et al. (2020), the MIC values of EOs were applied to 100 mL of 10^8 CFU/mL *E. coli* and *E. faecails* in Mueller Hinton broth, and the bacteria were then cultured at 37°C for three hours in an incubator shaker to investigate the effects of 1, 8-cineole on the bacterial ultrastructure. Cells that had no EO added were worked as a control group. The bacterial suspensions were centrifuged for 10 minutes at 4°C at 8000 rpm in sterile polypropylene centrifuge tubes. Following the supernatant's removal, the pelleted cellular material was fixed for an additional night at 4°C using 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH=7.4). After being post-fixed for two hours at room temperature in 1% osmium tetroxide (OsO4) dissolved in cacodylate buffer, samples were rinsed three times for fifteen minutes each in cacodylate buffer. The samples underwent two 15-minute cycles of ethanol dehydration after being diluted to 40%, 60%, 75%, 80%, and 95% in a graded order. An hour at 100% ethanol was necessary for the later step of dehydration, with adjustments made every thirty minutes. The post-fixed samples were implanted in epoxy resin (Epon-618) for 12 to 16 hours at 45°C. Ultra-thin sections of the embedded samples were prepared by an LKB-II Ultra-cut instrument and stained twice with lead citrate and uranyl acetate. Bacterial cell morphology was observed on a transmission electron microscope.

Statistical analyses

Every measurement and analysis was carried out three times. Using the Microsoft TM ExcelR application, fundamental statistical variability indicators were applied to the experimental data. The linear correlation analysis was used to express the dependency rate between the tested attributes.

Results and Discussion

Prevalence of bacterial isolates

The patients in this study ranged in age from 28 to 48, with the majority of them having infertility evaluation. Pyospermic semen made up about 40% of the semen samples. Sperm motility, count, and shape are all impacted by pyospermia (Farsimadan & Motamedifar 2020). In this respect, Nasrallah et al. (2018) reported that in about 43% of cases, pyospermia results in decreased fertility and poor-quality semen. According to Henkel et al. (2020), leukocytes negatively impact sperm function and fertilization rates. The urogenital tract inflammatory process is caused by bacteria and leukocytes found in semen; however, leukocytes seem to be the primary source of reactive oxygen species in seminal plasma and sperm suspensions. The prevalence rate of bacteriospermia in this work was 34.94%, comparable prevalence rates were previously recorded (Vilvanathan et al. 2016; Golshani et al. 2006). Higher prevalence rates of 52.5% and 68.79% were reported in other studies (Alekwe et al. 2023; Abbas et al. 2019). However, Domes et al. (2012) demonstrated a lower (15%) prevalence rate of bacteriospermia. In this respect, Tvrdá et al. (2022) recorded that, bacteriospermia impact the normal fertility process by the deterioration of spermatogenesis, reduced motility of sperm, altered morphology of sperm, reactive oxygen species formation, leading to increased DNA fragmentation index, anti-sperm antibodies formation causing obstruction of the genital tract due to inflammation. Table (1) indicates that out of 186 semen samples, 65 bacterial isolates were found, indicating a prevalence of 35.3% for bacteriospermia. The most frequently isolated organism was *Enterococcus faecalis* (38.5%), which was followed by *E. coli* (16.9%) and coagulase-negative *Staphylococcus haemolyticyus* (33.5%). *Micrococcus lylae* (4.6%) and *Serratia marcescens* (6.2%) were the less often isolated microbes. These outcomes are consistent with earlier published findings (Vilvanathan et al. 2016; Alekwe et al. 2023). In another study, Owolabi et al. (2016), stated that *Staphylococcus aureus* and *Staphylococcus epidermidis* are more frequently isolated.

Molecular characterization by 16S rRNA

The 16S ribosomal RNA (16S rRNA) sequencing is more accurate in the identification of Gram -ve and Gram +ve bacteria (Al-Buhamrah et al. 2024; Rawy et al. 2020; Kumar et al. 2020).The findings of 16S rRNA sequencing for the Gram-positive bacterial isolate in this investigation compared with the NSBI database using nucleotide blast to determine sequences that were 97% and 98% like standard strains of *S. haemolyticus* and *M. lylae*, respectively, Figures 1 and 2 displayed these

Table 1. Distribution of bacterial strains isolated from human semen samples

Bacterial isolates	Frequency	%
Gram positive		
Enterococcus faecails	25	38.46%
Staphylococcus haemolyticyus	22	33.84%
Micrococcus lylae	3	4.6%
Gram negative		
Escherichia coli	11	16.9%
Serratia marcescens	4	6.2%
Total count	65	100

Fig1. Phylogenetic tree, demonstrating the species affiliation of the isolated *S. haemolyticus* strain based on 16S rRNA gene sequences.

Fig 2. Phylogenetic tree, demonstrating the species affiliation of the isolated *M.lylae*strain based on 16S rRNA gene sequences.

Fig 3. Phylogenetic tree, demonstrating the species affiliation of the isolated *S. marcescens* strain based on 16S rRNA gene sequences.

Antibiotic susceptibility patterns of isolated bacteria

Table (2) displays the antibiotic susceptibility patterns of all bacterial isolates to common antibiotics. results. Additionally, a comparison of the Gram-ve bacterial isolate sequencing with the NSBI database (NCBI)revealed 98% similarity to standard *S. marcescens*(Figure 3). These outcomes concurred with recent studies (Al-brahim et al. 2023; Kosecka-Strojek et al. 2019). The *S. haemolyticus*, *M. lylae*, and *S. marcescens* strains were registered in the gen bank with accession numbers OR856634, OR856636, and OR856638 respectively. Both gram-positive and gram-negative demonstrated high resistance to widely used antibiotics tested. The most prevalent *E. faecalis* isolates were100% resistant to nalidixic acid, rifampin, amikacin, cefuroxime, and erythromycin while most of these strains were resistant to chloramphenicol, clindamycin, gentamicin, and ciprofloxacin (88 %) followed by penicillin-G (80 %) these in the agreement with El-Kattan et al. (2022).However, high degree of sensitivity rates (100%) to nitrofurantoin, vancomycin, trimethoprim/sulphamethoxazole, imipenem, meropenem, and piperacillin/tazobactam were found, similar results were previously reported (Abdelkareem et al. 2017; Chakraborty *et al*. 2015). Because *E. faecalis* can acquire and spread antibiotic-resistant genes, it has developed drug resistance (El-Kattan & Allam 2021). 100% of *S. haemolyticus* were resistant to cefuroxime and clindamycin, and 95.4% of isolates were resistant to erythromycin. Also, 90% of isolates found resistance to nitrofurantoin, rifampicin, and penicillin-G. In addition, *S. haemolyticus* demonstrated great sensitivity to other antibiotics. These findings are in agreement with previous studies **(**Kalaiselvan et al. 2022; Panda et al. 2016). *S. haemolyticus* is mainly multidrug-resistant and produces enzymes and toxins that result in difficult-to-treat infections (Eltwisy et al. 2022). In this study, 100% of *M. lylae* strains represented resistance to penicillin-G, nitrofurantoin, cefuroxime, clindamycin, and erythromycin. Otherwise, other antibiotics were active with *M. lylae* strains, these results are in agreement with Sapkota *et al*. (2020). *M. lylae* isolates are opportunistic pathogens related to various infections and demonstrated resistance to multiple antibiotics (Munawar et al. 2021). 100% of the *S. marcescens* were resistant to cefuroxime, clindamycin, vancomycin, penicillin-G, and erythromycin. Additionally, 100% of the isolates were sensitive to the most tested antibiotics. These results line up with earlier studies (Liou et al. 2014; Özcan et al. 2020). All *E. coli* isolates demonstrated resistance to cefuroxime, clindamycin, vancomycin, penicillin-G, and erythromycin, furthermore, variable resistance was found to rifampin (80%), gentamicin (60%), and piperacillin/tazobactam (60%). In addition, all isolates of *E.coli* were completely sensitive to nalidixic acid,

levofloxacin, trimethoprim/sulphamethoxazole, imipenem, and ciprofloxacin. Similar results were registered in previous studies (El-Kattan & Allam 2021; Kazemnia et al. 2014). In constant with Goda *et al*. (2023), all antimicrobials, except carbapenem, showed multipleantimicrobial resistances. High levels of antibiotic resistance may be caused by the antibiotics that are frequently used in empirical treatment (Salam et al., 2023), the resistance mechanism may be attributed to several genetic factors; mutations, cell wall ticking, production of ß-lactamase, and modification of the site(s) receptors specific for antibiotics (Hassan and Motaweq 2024; Munita & Arias 2016).

Antimicrobial effects of essential oils

The antimicrobial activity results of three EOs against bacteria isolated from human semen using levofloxacin as a negative control and amikacin as a positive control are recorded in Table (3), and figures 4 and 5.All bacteria were susceptible to three EOs even in the lowest amounts. In the current investigation, EOs showed strong antibacterial activity when compared to control. However, the CEO demonstrated inhibitory effects with all different concentrations.The strongest antibacterial effect was against *M*.lylae (39.7mm) following this *S. haemolyticus* (34.3 mm), *E. coli* (22.7 mm), *E. faecalis* (21.3mm) and *S. marcescens* (17.3 mm), these results are relatively in accordance with the previous reports (Coșeriu et al. 2023; Patterson et al. 2019; Karadağlıoğlu et al. 2019; Clemente et al. 2016). As stated in a previous study by El-Kattan and Allam (2021), the high antibacterial activity observed with cinnamon EO may be because of the action of trans-cinnamaldehyde, this compound may be disrupting the cell membrane of bacteria leading to cell death. Moreover, rosemary EO exhibited various activities against all tested bacteria with different inhibition zones. The highest zones were 23.3mm, 19.3mm, 17.3mm, 12.7mm, and 12.3mm against *M. lylae*, *S. haemolyticus*, *E. faecalis*, *S. marcescens* and *E. coli* respectively, these findings are in accordance with the previous studies (Yeddes et al. 2019; Duckova et al. 2012). Rosemary EO has been widely used in traditional medicine and cosmetics. According to Adriana et al. (2013), the antibacterial activity of rosemary essential oil against both Gram-positive and Gram-negative bacteria was found to be related to its α-pinene content, the potent antibacterial, antioxidant, cytotoxic, and antimutagenic qualities of this substance have contributed to medicinal applications (Stojiljkovic et al. 2018). Furthermore, lavender EO was found to have distinct inhibitory effects with inhibition zones of 37.3 mm, 17.7 mm, 17.3 mm, 15.7 mm, and 15.0 mm against *M. lylae*, *E. faecalis*, *S. marcescens*, *E. coli*, and *S. hemolyticus*, respectively. These findings have been supported by other studies (Gismondi et al. 2021; Fathima et al. 2015; Sienkiewicz et al. 2014). Based on earlier studies (Hossain et al. 2017), LEO had significant antibacterial activity because of the high concentration of active phenolic components including Linalool. Ahmad et al. (2019) recorded that Linalool is the most potent major constituent component in LEO. It should be mentioned that every tested strain in this study that was sensitive to EOs also displayed resistance to commercial antibiotics. Amer et al. (2024) indicated that essential oils exhibit antimicrobial properties against a diverse array of bacteria, including those that are resistant to multiple drugs. According to Valková et al. (2021), the variations in the sensitivity of bacteria to EOs depend on the nature of the EOs used, their concentration, and the kind of tested microorganism.

Minimum inhibitory concentration values

The evaluated essential oils' minimum inhibitory concentrations (MIC) against both Gram-positive and Gram-negative bacteria are shown in Table (4). Varying values of minimum inhibitory concentrations (MIC) are based on the bacterial strains and essential oils. The MIC values of cinnamon EO were determined to be 15.6 and 31.2 µL/mL. In addition, the MIC of lavender and rosemary EOs ranged from 15.6 to 125 µL /mL. These results corroborated with previous studies (Diass et al. 2023; Jafari-Sales et al. 2020; Sim et al. 2019) but, they are lower than those determined in earlier studies (Denkova et al. 2023; Elyemni et al. 2022; Puvača et al. 2021; Chouhan et al. 2017).

Table 2. Antibiotic resistance and sensitivity (%) of bacterial strains isolated from human semen

Antibiotic discs	*Bacterial strains*									
	E. faecalis		***S.haemolyticus***		***M.lylae***		***S. marcescens***		***E. coli***	
	R	S	R	S	R	S	R	S	R	S
Chloramphenicol (30 µg)	88	12	0	100	0	100	0	100	27.3	72.7
Nalidixic acid (10 µg)	100	0	0	100	0	100	0	100	0	100
Nitrofurantoin (10 µg)	0	100	90	10	100	0	50	50	27.3	72.2
Rifampicin (30 µg)	100	0	90	10	33.3	66.7	50	50	90.9	9.1
Amikacin (10 µg)	100	0	4.6	95.4	0	100	25	75	36.4	63.6
Cefuroxime (30 µg)	100	0	100	0	100	0	100	0	100	0
Clindamycin (30 µg)	88	12	100	0	100	0	100	0	100	0
Vancomycin (5 µg)	0	100	0	100	0	100	100	0	100	0
Penicilin-G (10 µg)	80	20	90	10	100	0	100	0	100	0
Tetracycline (10 µg)	52	48	9.1	90.9	33.3	66.7	50	50	9.1	90.9
Levofloxacin (10 µg)	48	52	0	100	0	100	0	100	0	100
Gentamicin (5 µg)	88	12	13.6	86.4	33.3	66.7	25	75	63.6	36.4
Trimethoprim/sulphamethoxazole (30 µg)	0	100	0	100	0	100	0	100	0	100
Imipenem (10µg)	0	100	0	100	0	100	0	100	0	100
Erthromycin(10µg)	100	0	95.4	4.6	100	0	100	0	100	0
Meropenem (10µg)	0	100	0	100	0	100	0	100	0	100
Ciprofloxacin (10µg)	88	12	0	100	0	100	0	100	0	100
Piperacillin/tazobactam (30 µg)	0	100	4.6	95.4	0	100	0	100	63.6	36.4

The percentage (%) of bacterial resistance (R) and bacterial sensitivity (S)

Table 3. Antimicrobial activity of different concentration of essential oils against bacterial isolates

Isolates	Essential oil										
	Cinnamon			Rosemary			Lavender			Control	
	Mean of zone inhibition in mm (mean ± SD)										
	1:1	1:2	1:4	1:1	1:2	1:4	1:1	1:2	1:4	+Ve	-Ve
E. faecalis	21.3 ± 0.57	12.3 ± 0.57	10.0 ± 0.0	17.3 ± 0.57	14.3 ± 0.57	11.7 ± 0.57	17.7 ± 0.57	11.7 ± 0.57	10.0 ± 0.0	17.0 ± 0.0	_
E. coli	22.7 ± 0.57	19.0 ± 0.0	10.7 ± 0.57	12.3 ± 0.57	11.0 ± 0.0	9.0 ± 0.0	15.7 ± 0.57	14.0 ± 0.0	11.3 ± 0.57	17.3 ± 0.57	_
M. lylae	39.7 ± 0.57	34.3 ± 0.0	17.0 ± 0.0	23.3 ± 0.57	27.7 ± 0.57	21.7 ± 0.57	37.3 ± 0.57	30.3 ± 0.57	21.3 ± 0.57	32.0 ± 0.0	_
S.marcescens	17.3 ± 0.57	15.0 ± 0.0	10.7 ± 0.57	12.7 ± 0.57	10.7 ± 0.57	9.3 ± 0.57	17.3 ± 0.57	14.7 ± 0.57	9.3 ± 0.57	15.0 ± 0.0	_
S.haemolyticus	34.3 ± 0.57	27.7 ± 0.57	22.3 ± 0.57	19.3 ± 0.57	14.7 ± 0.57	9.7 ± 0.57	15.0 ± 0.0	12.3 ± 0.0	9.7 ± 0.57	17.3 ± 0.57	_

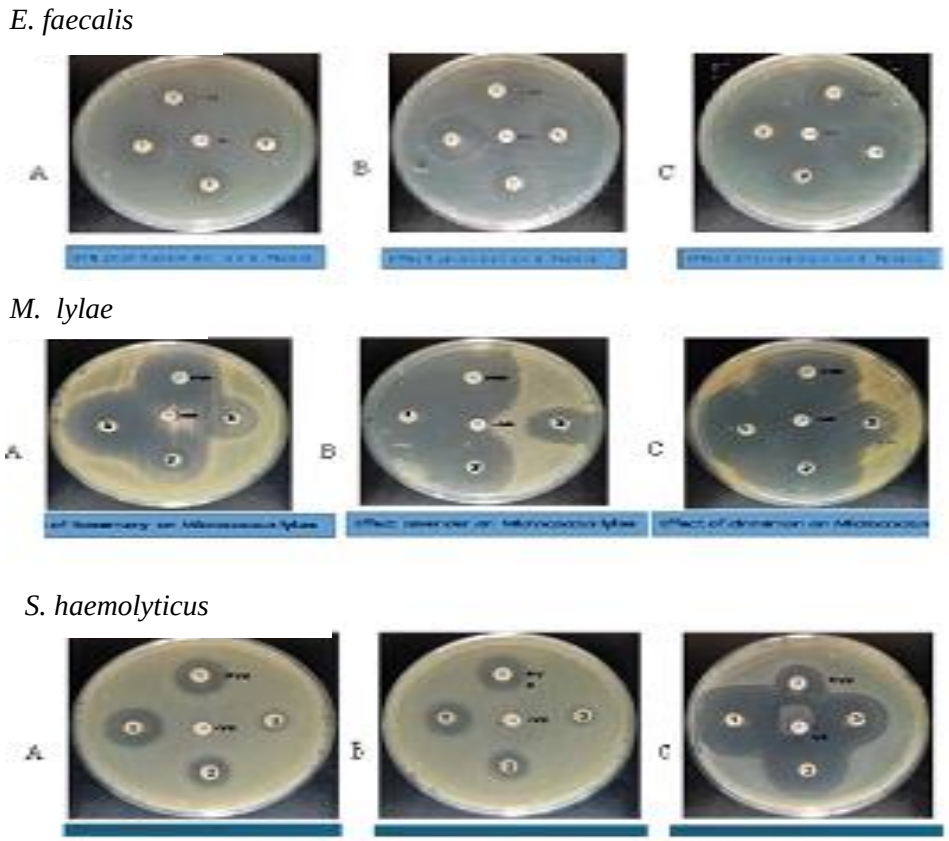

Fig 4. Antimicrobial activity of different concentrations of REO (**A**); LEO (**B**); CEO (**C**) against gram-positive bacteria, *E. faecalis*, *M. lylae*, and *S. haemolyticus* using levofloxacin as a negative control (–ve) and amikacin as a positive control (+ve).

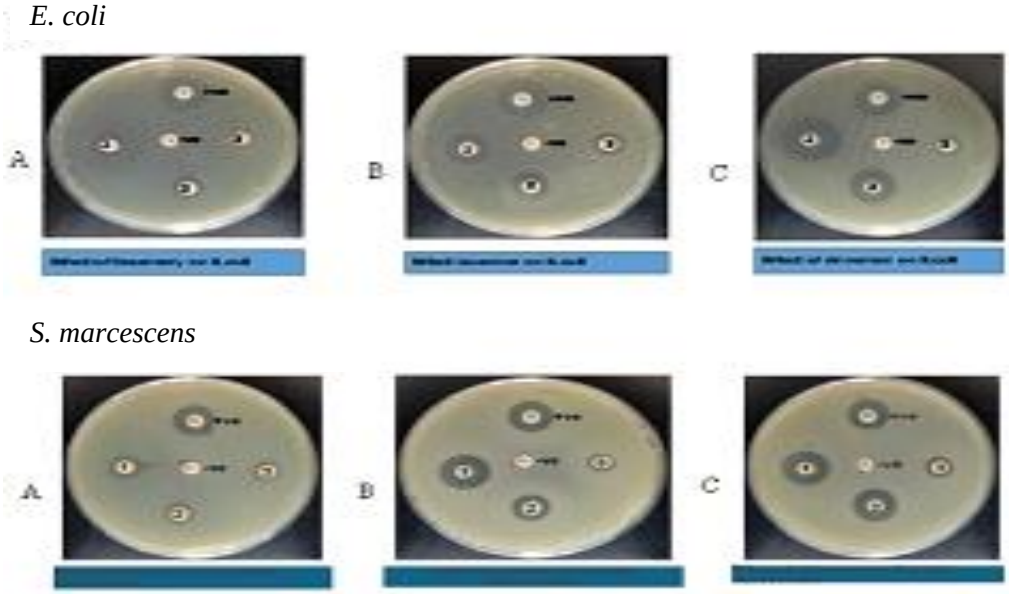

Fig 5. Antimicrobial activity of different concentrations of REO (A); LEO (B); CEO (C) against gram-negative bacteria, *E. coli*, and *S. marcescens* using levofloxacin as a negative control (–ve) and amikacin as a positive control (+ve).

Table 4. Minimum inhibitory concentrations (µL/mL) of essential oils against bacterial isolates

Bacterial isolates	Essential oil		
	Cinnamon	Rosemary	Lavender
	MIC µL/mL		
E. faecalis	31.2	31.2	15.6
E. coli	31.2	31.2	62.5
M. lylae	15.6	31.2	15.6
S. marcescens	31.2	125	62.5
S. haemolyticus	15.6	125	125

GC-MS Analysis results of studied essential oils

The ingredient constituents of cinnamon, rosemary, and lavender EOs were identified by GC-MS analysis (gas chromatography-mass spectrometry) analysis (Figures 6, 7, 8). GC-MS analysis identified a total of 15 compounds in cinnamon EO representing 100 % of the total constituents as present in Table (5). Each component was displayed along with their retention time and concentration (%). The dominant compounds were Cinnamaldehyde, (E) (43.55%), followed by, Cinnamaldehyde dimethyl acetal (38.97%) and Glycerol diacetate (14.64%). These findings suggest that the main constituents in the CEO were aldehydes. Similar findings were found by Yu et al. (2020). The flavor of cinnamon is mostly attributed to cinnamic aldehyde (Rao and Gan 2014), which also has potent antibacterial qualities against gram +ve and gram -ve pathogenic bacteria (Nabavi et al. 2015). Additionally, GC-MS analysis revealed 13 compounds in lavender essential oil, accounting for 100% of all constituents; the results are shown in Table (6), the most commonly found chemicals were Linalool (49.45%), Terpinyl acetate (32.98%), and Camphor (7.97%). In this respect, Singh et al. (2015) recorded that, Linalyl acetate (39.10%), Linalool (29.7%), and α -terpineol (4.35%) were found as the main ingredients in lavender oil. According to Beale et al. (2017), the majority of oxygenated monoterpenes were produced by lavender, followed by monoterpene hydrocarbons with Linalool as the active ingredient. Additionally, *Lavandula angustifolia* is medicinally used all over the world for its broad biological as well as pharmaceutical uses against various diseases or pathogens (Stierlin et al. 2020). In this work, GC-MS analyses of rosemary essential oil are given in Table (7), where the identified compounds represented 100 % of the ingredients. The main constituents were Limonene **(**34.99%), followed by Linalool (23.89%), Camphor (17.86%), and Caryophyllene (9.27%). All these compounds of *Rosmarinus officinalis* were reported in recent studies as having antimicrobial activities; however, most researchers focused on the separate effect of each compound (Behiry et al. 2022; El-Nagar et al. 2020). According to Rahbardar and Hosseinzadeh (2020)**,** REO has been employed as a medicinal agent to treat several diseases.

Table 5: Main compounds of the cinnamon essential oil identified by GC/MS

Peak	RT	Compound Name	Probability	Formula	MW	Area %
1	10. 74	1-(3-Oxobutyl)-3,3-dimethyldiaziridine	8.09	C7H14N2O	142	0.16
2	12. 46	1,5-Heptadien-4-ONE, 3,3,6-trimethyl	12.32	C10H16O	152	0.26
3	15. 89	1-Propanol, 2-(2-hydroxypropoxy)	26.62	C6H14O3	134	0.17
4	26. 37	Cinnamaldehyde, (E)	29.63	C9H8O	132	43.55
6	29. 83	1,2,3-Propanetriol, triacetate	36.97	C9H14O6	218	0.24
7	29. 93	Glyceryl diacetate (Glycerol 1,2-diacetate)	40.16	C7H12O5	176	14.64
8	32. 01	Cinnamaldehyde dimethyl acetal	57.32	C11H14O2	178	38.97
9	37. 45	7-Methyl-1-naphthol	12.65	C11H10O	158	0.14
10	51. 39	Hexadecanoic acid, methyl ester	72.00	C17H34O2	270	0.64
11	53. 70	2-Propenoic acid, 3-phenyl-, 2-methylpropyl ester	9.66	C13H16O2	204	0.08
12	53. 87	n-Propyl cinnamate	18.39	C12H14O2	190	0.11
13	57. 71	Methyl stearate	70.31	C19H38O2	298	0.68
14	63. 16	Glycidyl palmitate	78.07	C19H36O3	312	0.18
15	76. 20	13-Docosenamide, (Z)-	47.05	C22H43NO	337	0.18

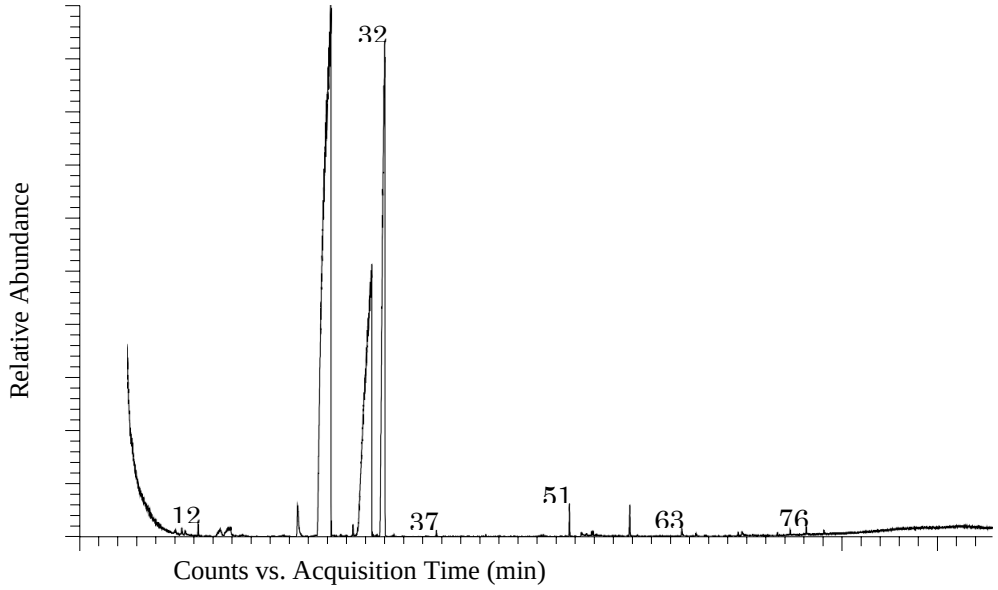

Fig 6. GC-MS/MS Chromatogram of all Identified Compounds in cinnamon EO.

Table 6: Main compounds of the Lavender essential oil identified by GC/MS

Peak	RT	Compound Name	Probability	Formula	MW	Area %
1	13. 79	Limonene	21.56	C10H16	136	1.81
2	15. 00	p-Cymene	23.61	C10H14	134	1.31
3	20. 16	Linalool	58.07	C10H18O	154	49.45
4	20. 87	Camphor	12.07	C10H16O	152	7.97
5	27. 09	á-Terpinyl acetate(Terpinyl acetate)	49.58	C12H20O2	196	32.98
6	27. 36	Pentanenitrile, 2-(aminomethylene)-3, 3-dimethyl-, (Z)	66.45	C8H14N2	138	1.6
7	29. 60	(Citronellic acid)	18.80	C10H18O2	170	2.61
8	32. 52	Longifolene	12.06	C15H24	204	0.27
9	35. 74	9-(3,3-Dimethyloxiran-2-yl)-2,7 -dimethylnona-2,6-dien-1-ol	12.13	C15H26O2	238	0.24
10	51. 40	Hexadecanoic acid, methyl ester	69.72	C17H34O2	270	0.46
11	57. 71	Methyl stearate(Octadecanoic acid)	64.79	C19H38O2	298	0.45
12	76. 22	(E)-3,7-Dimethylocta-2,6-dien -1-yl palmitate(Ethylhexyllinolate)	27.75	C26H48O2	392	0.52
13	80. 93	(Linoleylcaprat)	31.71	C28H52O2	420	0.33

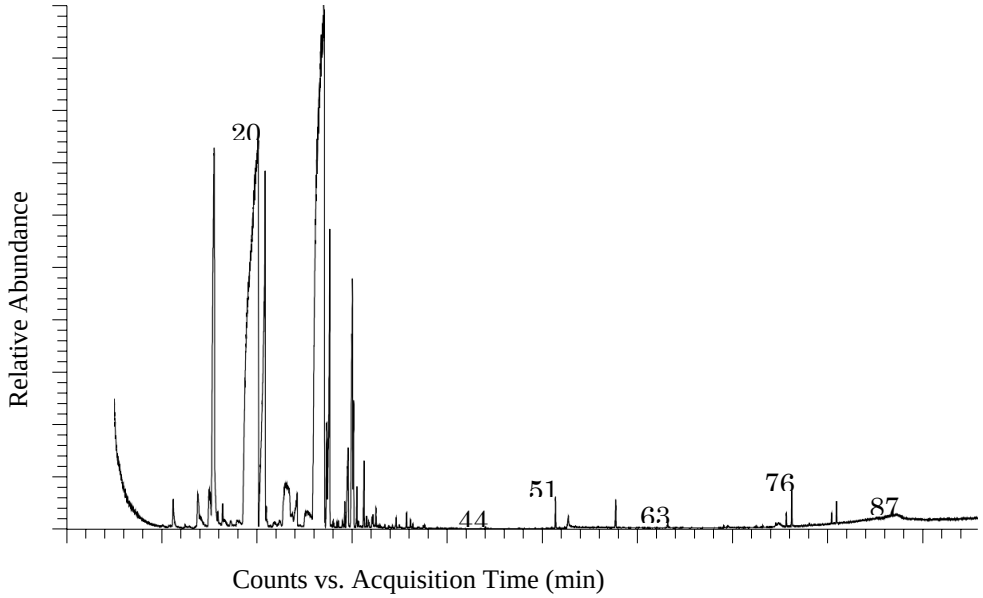

Fig 7. GC-MS/MS Chromatogram of all Identified Compounds in lavender EO.

Table 7: Main compounds of the Rosemary essential oil identified by GC/MS

Peak	RT	Compound Name	Probability	Formula	MW	Area %
1	12. 08	Limonene	26.70	C10H16	136	34.99
2	12. 19	Bicyclo[3.1.0]hex-2-ene, 4-methylene-1-(1-methylethyl)	57.91	C10H14	134	1.67
3	15. 93	Linalool	62.10	C10H18O	154	23.89
4	20. 73	Terpenoid(Camphor)	31.00	C10H16O	152	17.86
5	21. 20	Pinocarvone(Carvacrol)	29.74	C10H14O	150	3.12
6	23. 41	5,9,9-Trimethyl-spiro[3.5]non-5 -en-1-one	9.51	C12H18O	178	1.65
7	23. 75	Bicyclo[3.1.1]hept-2-ene -2-ethanol, 6,6-dimethyl	84.71	C11H18O	166	0.56
8	27. 23	Bornyl acetate	23.12	C12H20O2	196	3.95
9	29. 75	3-Allyl-6-methoxyphenol	18.73	C10H12O2	164	0.79
10	36. 77	Caryophyllene	25.95	C15H24	204	9.27
11	39. 15	(-)-5-Oxatricyclo[8.2.0.0 (4,6)]dodecane,,12-trime thyl-9-methylene-, [1r-(1r*,4r*,6r*,10s*)]	77.60	C15H24O	220	1.68
12	41. 28	.tau.-Cadinol	43.29	C15H26O	222	0.57

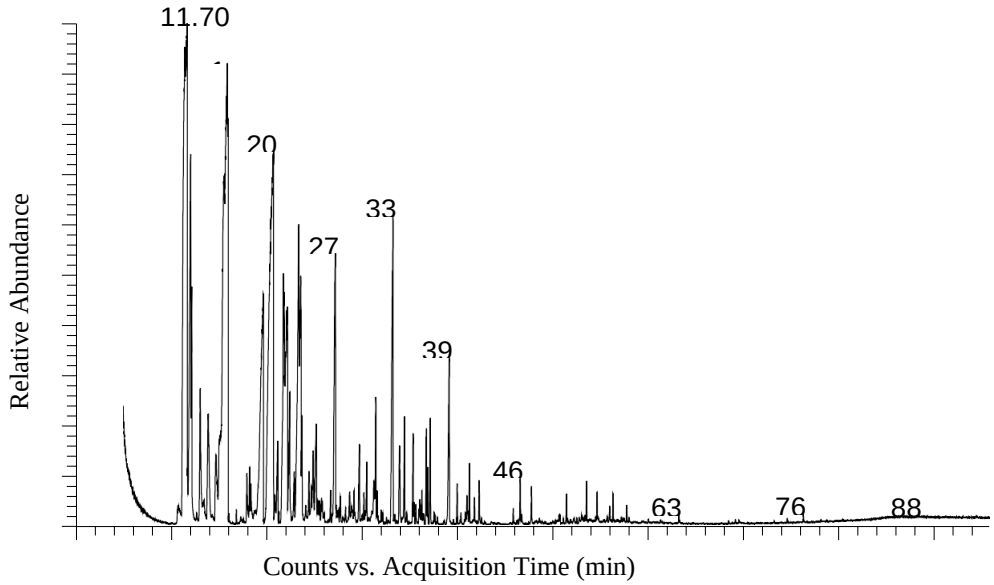

Fig 8. GC-MS/MS Chromatogram of all Identified Compounds in rosemary EO.

The effect of essential oils on bacterial cells under the transmission electron microscope

Figures 9 and 10 showed the *E. coli* and *E. faecalis* cells treated with CEO, LEO, and REO were observed with a transmission electron microscope. The treated *E. coli* cells had substantial damage, with split cell walls and membranes and reduced nucleoplasm (Figure 9 b-d). In contrast, the non-treated *E. coli* cells had a healthy shape of bacilli (Figure 9 a). The smooth cell wall, cell membrane, and nuclear material of the untreated *E. faecalis* cells were completely normal (Figure 10 a). However, following EO treatment, *E. faecalis* showed alterations in size and shape as well as a decrease in cytoplasm (Figure 10 b-d). The active ingredients in EOs can readily enter and stack in the lipid layer of cytoplasmic membranes (Yammine et al. 2022). According to El-Tarabily et al. (2021), the highest antibacterial properties of EOs are attributable to their high phenol and aldehyde content, which increase cell permeability and cause cell leakage, disrupting normal membrane function. Most EOs break DNA, release lipopolysaccharides, alter the ATP balance, and cause cytoplasmic changes (Ellboudy et al. 2023).

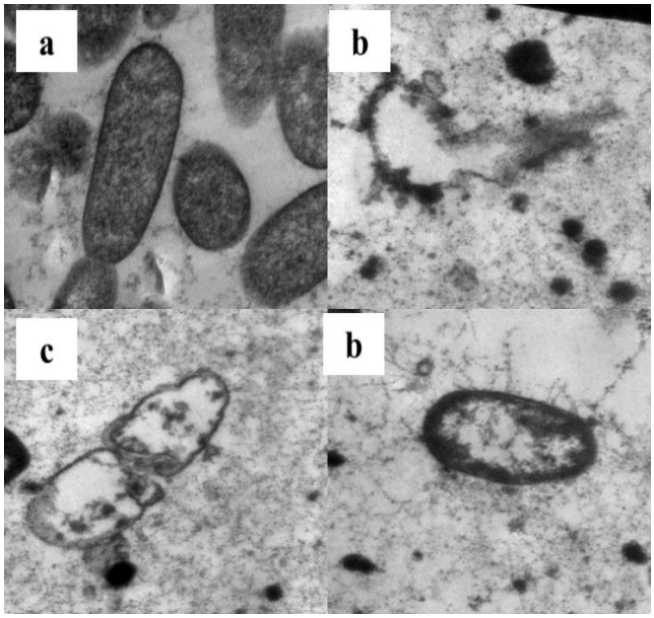

Fig 9.TEM graph (×25000) reveals the effect of three EO on *E. coli* (non-treated) (**a**), *E. coli* exposed to CEO (**b**), *E. coli* exposed to LEO (**c**), *E. coli* exposed to REO (**d**).

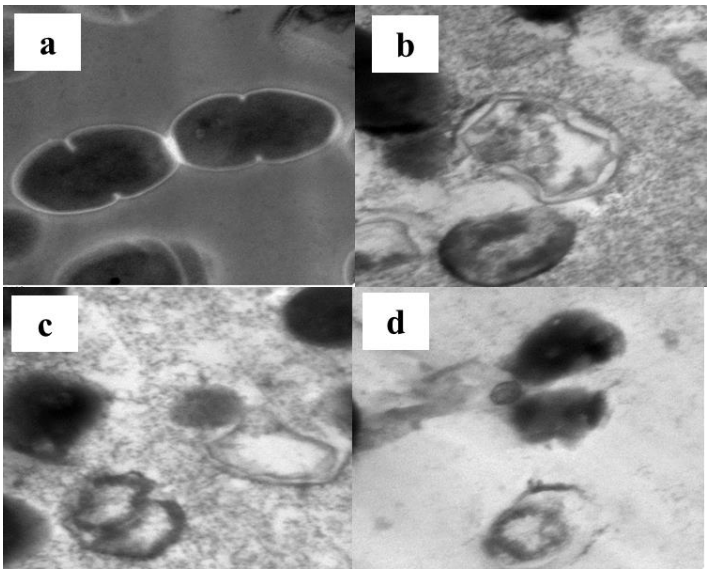

Fig 10. TEM graph (×25000) reveals the effect of three EO on *E. faecalis* (non-treated) (**a**), *E. faecalis* exposed to CEO (**b**), *E. faecalis* exposed to LEO (**c**), *E. faecalis* exposed to REO (**d**).

Conclusion

The findings suggestthat the essential oils of rosemary, lavender, and cinnamon have demonstrated good potential as natural antibacterial agents. These essential oils have shown significant antibacterial properties against *E. faecails*, *S. haemolyticus*, *E. coli*, *M. lylae*, and *S. marcescens*, which cause pyospermia. The GC-MS analysis revealed the presence of active compounds such as Cinnamaldehyde, (E) (43.55%), Linalool (49.45%), and Limonene (34.99%),which possess strong antibacterial activity against (Gram +ve) and (Gram -ve) pathogenic bacteria. These active ingredients in EOs can increase cell permeability and cause cell leakage and reduce nucleoplasm. Therefore, it can be said that the CEO, LEO, and REO are good sources of natural substances and could be a good alternative to artificially produced antimicrobials.

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Conflict of interest

The authors declare that they have no conflict of interest.

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